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Maternal-fetal cholesterol transport in the second half of mouse pregnancy does not involve LRP2

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Abstract

Aim

Lipoprotein related receptor protein type 2 (LRP2) is highly expressed on both yolk sac and placenta. Mutations in the corresponding gene are associated with severe birth defects in humans, known as Donnai-Barrow syndrome. We here characterized the contribution of LRP2 and maternal plasma cholesterol availability to maternal-fetal cholesterol transport and fetal cholesterol levels *in utero* in mice.

Methods

Lrp2^{+/-} mice were mated heterozygously to yield fetuses of all 3 genotypes. Half of the dams received a 0.5% probucol enriched diet during gestation to decrease maternal HDL cholesterol. At E13.5 the dams received an injection of D7 labeled cholesterol and were provided with 1-¹³C acetate-supplemented drinking water. At E16.5, fetal tissues were collected and maternal cholesterol transport and fetal synthesis quantified by isotope enrichments in fetal tissues by GC-MS.

Results

The *Lrp2* genotype did not influence maternal-fetal cholesterol transport and fetal cholesterol. However, lowering of maternal plasma cholesterol levels by probucol significantly reduced maternal-fetal cholesterol transport. In the fetal liver, this was associated with increased cholesterol synthesis rates. No indications were found for an interaction between the *Lrp2* genotype and maternal probucol treatment.

Conclusions

Maternal-fetal cholesterol transport and endogenous fetal cholesterol synthesis depend on maternal cholesterol concentrations but do not involve LRP2 in the second half of murine pregnancy. Our

results suggest that the mouse fetus can compensate for decreased maternal cholesterol levels. It remains a relevant question how the delicate system of cholesterol transport and synthesis is regulated in the human fetus and placenta.

Key words: lipoprotein, placenta, transport, fetus, cholesterol

Introduction

Cholesterol is important for embryonic development and fetal growth, since it is part of all cell membranes, a precursor for steroid hormones, and the activator of the sonic hedgehog (SHH-GLI) transcription pathway. Besides cholesterol synthesis by the fetus itself, it has been shown that maternal and fetal plasma cholesterol levels are strongly correlated during most of gestation¹ and recent human studies have indicated that during the second trimester of pregnancy maternal cholesterol is transported to the fetus in significant amounts^{2,3}. This suggests that maternal cholesterol may be the most important source for the growing fetus during organ development, when its own cholesterol synthesis is relatively low.

Defects in cholesterol synthesis pathways, like in Smith-Lemli-Opitz syndrome or in desmosterolosis, are characterized by a range of congenital birth defects, both in humans and murine models^{4,5}. Similarly, errors in the SHH-GLI transcription pathway are known to cause serious birth defects, ranging from mild microcephaly to severe holoprosencephaly, neural tube defects and heart defects⁶⁻⁸. It is therefore not surprising that associations between low maternal cholesterol levels and adverse birth outcome have previously been described in humans⁹⁻¹¹. Recently, a study showed that also mothers with increased cholesterol levels have a higher chance of giving birth to a child with a congenital heart defect¹². Analyzing the effects of hypercholesterolemia in pregnant women is more complex since cholesterol levels are not routinely screened for during pregnancy. It is however of crucial importance to know whether maternal-fetal cholesterol transport is involved in

organogenesis since the prevalence of diseases affecting maternal cholesterol levels such as diabetes and obesity are increasing rapidly among pregnant women¹³.

Maternal-fetal interfaces - the yolk sac and placenta - are known to express a large number of proteins involved in cholesterol transport, which further suggests that maternal cholesterol is an important source of fetal cholesterol (reviewed by:^{14,15}). Absence of these cholesterol transporters in mouse models show different phenotypes, ranging from early developmental arrest and exencephaly in scavenger receptor class B deficient embryos¹⁶, to no birth defects in low density lipoprotein receptor knockout pups¹⁷.

One receptor is, however, of particular interest since it is known that mutations in this gene give a human phenotype with congenital anomalies, the Donnai-Barrow syndrome¹⁸: Lipoprotein receptor related protein 2 (LRP2), also known as megalin, is highly expressed on both secondary yolk sac and placenta during pregnancy (reviewed in¹⁹). It is known to bind a large variety of ligands, including LDL cholesterol and, in co-transport with cubilin, is furthermore involved in HDL cholesterol transport²⁰. Mice homozygous for *Lrp2* knockout are born with severe birth defects; including holoprosencephaly, neural tube defects and kidney abnormalities, and die within 24 hours after birth²¹. Although absence of functional LRP2 in the affected fetal organs itself is the most likely cause of the observed congenital anomalies of *Lrp2*^{-/-} fetuses, a contributing effect of receptor function on nutrient transport in the placenta and the yolk sac is likely^{22,23}.

We have very recently described that *Lrp2*^{-/-} fetuses develop severe congenital heart anomalies *in utero*²⁴. *Lrp2*^{-/-} fetuses show high penetrance of congenital heart anomalies, including common arterial trunk, aortic arch anomalies, straddling tricuspid valve and marked thinning of the ventricular myocardium. We here performed a cholesterol transport study in *Lrp2*^{-/-} mice to answer the question whether LRP2 on yolk sac and placenta is substantially involved in maternal-fetal cholesterol transport and to what extent this contributes to organ development.

[25,26,26,26,27,27,27]-D7-cholesterol was injected in the dams, followed by administration of 1-¹³C

labeled acetate in the drinking water. Based on the distribution of the labels in different tissues and incorporation of the acetate in newly formed cholesterol, both transport and synthesis rates could be calculated (Mass Isotopomer Distribution Analysis (MIDA)). As HDL and LDL particles are known LRP2 ligands, we hypothesized that a lack of maternal cholesterol could be contributing to the pathogenesis of the congenital heart anomalies in *Lrp2* knockout embryos.

To further elucidate the effect of adverse maternal cholesterol levels on maternal-fetal cholesterol transport and resulting cholesterol levels in the mouse fetus, we administered probucol to half of the dams during pregnancy. Probucol administration in mice is associated with a 60% reduction in total plasma cholesterol, mainly by reduction of HDL cholesterol levels²⁵. One of the mechanisms by which probucol lowers plasma cholesterol levels is by inhibition of the ATP binding cassette transporter A1 (ABCA1), which is crucially involved in the formation of HDL²⁶. Analyzing the effects of reduced HDL levels on maternal-fetal transport may be of particular interest since maternal obesity and diabetes are known to lead to a reduction in HDL cholesterol, which may affect maternal-fetal lipid exchange characteristics²⁷.

In addition, the ABC transporters *Abca1* and *Abcg1* are expressed in multiple compartments of the placenta, indicative for a synergistic function in lipid exchange during pregnancy. Although the exact mechanisms of action and directionalities of transplacental lipid exchange through these two transporters are not fully understood, a role for ABCA1 and ABCG1 in mediating efflux of cholesterol from human fetal endothelial cells to the fetal circulation has been suggested previously^{28,29}.

In order to quantify the effect of *Lrp2* genotype and probucol administration on maternal-fetal cholesterol transport and fetal cholesterol levels *in utero*, *Lrp2* mice were bred heterozygously while receiving either a control or probucol supplemented diet. Stable isotope labelling was performed to quantify maternal-fetal cholesterol transport and fetal cholesterol synthesis in fetuses of different

Lrp2 genotypes. Together, the combination of genotype and lowered maternal cholesterol availability by administration of probucol allowed us to determine the role of LRP2 in maternal-fetal cholesterol transport.

Materials and methods

Animals

Mice with a disruption of the *Lrp2* gene have been described previously²¹ and were kindly provided by Prof. Thomas Willnow (Max Delbrück Center for Molecular Medicine, Berlin, Germany). Female *Lrp2*^{+/-} mice were fed either RMH-B breeding and maintenance diet (AB-diets, Woerden, Netherlands; containing 5% fat, 23.5% protein, 42.3% carbohydrates), or the former supplemented with 0.5% probucol (Sigma-Aldrich, Zwijndrecht, Netherlands). The animals were housed in temperature controlled rooms (23°C) with a 12/12h light/dark cycle. Food and water were provided *ad libitum* throughout the experiments. After one week, the *Lrp2*^{+/-} females were mated to *Lrp2*^{+/-} males overnight, the morning after the mating was taken as E0.5. At the time of mating, females were between 10 and 16 weeks of age. All experimental procedures have been performed under the consent of the local ethical committee for animal experiments of the University of Groningen.

Experimental Procedures

Administration of stable isotopes and fetal tissues collection

On embryonic day 13.5 (E13.5), the pregnant females received a 0.5 mg intravenous dose of [25,26,26,26,27,27,27]-D7-cholesterol (Cambridge Isotope Laboratories, Andover, MA) dissolved in 20% intra-lipid (Fresenius Kabi, Den Bosch, Netherlands) by retro-orbital injection. Directly following the injections, the females were housed separately and provided with 2% 1-¹³C labeled acetate

(Isotec, Miamisburg, OH) supplemented drinking water. Bloodspots were collected before administration of the labeled cholesterol/acetate (T=0) and twice a day, by tail bleeding on filter paper. At embryonic day 16.5, the females were anaesthetized with isoflurane and terminated by cardiac puncture. Blood was collected in EDTA containing tubes; the plasma fraction was collected after 15 minutes centrifugation at 2000 g at 4°C, and finally stored at -80 °C.

Uteri of the pregnant dams were excised and kept in PBS on ice while extracting the fetuses. Fetal blood was collected as spots by exsanguination on filter paper. In addition, a small amount of fetal blood was collected using heparinized capillaries to obtain fetal plasma for the determination of cholesterol concentrations. Both for the stable isotope data and RT-qPCR analysis, the fetal part of the placenta was isolated by removal of maternal *decidual* tissue after excision from the *uterus*. Fetal liver, brain, the remaining carcass (from here one referred to as “fetal body”) and placenta’s were then collected in pre-weighed tubes, snap-frozen in liquid nitrogen and stored at -80°C for isotope analysis,.

For genotyping, genomic DNA was isolated from fetal tail tip using the Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich) following standard manufacturers’ protocol. The WT allele was amplified by the primers Lrp2-E+T-F (5’-CATATCTTGGAATAAAGCGACATC-3’) + Lrp2-E-R (5’-TCTCCTGTCTAGTCCCATCGTA-3’), producing a 361 bp fragment. Detection of the mutant allele was performed using the primers Lrp2-E+T-F + Lrp2-T-R (5’-GAGGATTGGAAGACAATAGCA-3’), resulting in a 300 bp product. The PCR program utilized was 95 °C for 10 min., followed by 32 cycles of 95 °C for 45s, 60 °C for 45s, and 72 °C for 45s, with a final step of 7 min. at 72 °C.

Histological examination of the fetal hearts

For histological examination of the fetal hearts, we isolated thoraxes of the embryos at E15.5. They were fixed in 4% paraformaldehyde for 48 hours and routinely processed for paraffin immunohistochemical evaluation, using myocardial light chain-2a (Mlc-2a) as a myocardial marker. Counterstaining was performed with 0.1% haematoxylin (Merck, Darmstadt, Germany) for 5 sec,

followed by rinsing with tap water for 10 min, dehydration and finally, mounted with Entellan (Merck, Darmstadt, Germany). To examine the effect of low maternal cholesterol on cardiac development in heterozygous fetuses, a total of 12 *Lrp2*^{+/-} fetuses were examined both by histological examination as well as cardiac morphometry. We performed morphometry on the ventricular myocardium at E15.5 by analyzing the ratio of the compact ventricular myocardial volume against the total ventricular myocardial volumes. For an extensive description of the methodology see Gundersen et al.³⁰.

Analytical procedures

FPLC

Pooled plasma samples from dams of the control and probucol supplemented group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden). Samples were chromatographed at a flow rate of 0.5 ml/min and fractions of 500 µl were collected. Individual fractions were assayed for cholesterol concentrations using a commercially available enzymatic assay (Roche Diagnostics, Mannheim, Germany).

Lipid extractions and cholesterol determination

Cholesterol was extracted from both dam and fetal bloodspots with 1 ml of 100% ethanol/acetone (1:1 v/v) for gas chromatography/mass spectrometric analysis according to³¹. Prior to the lipid extractions, 10% PBS-tissue homogenates were obtained from whole tissue using a Tissuelyser LT (Qiagen, Hilden, Germany), followed by total lipid extraction according to Bligh and Dyer³². A subfraction of the total lipid tissue extracts was emulsified using 2% triton X-100, after which cholesterol concentrations were determined using a commercial available enzymatic assay (Roche Diagnostics).

Gas chromatography/mass spectrometry (GC/MS)

For GC/MS, unesterified cholesterol from both bloodspots and tissue extracts was derivatized using N,O-bis-(trimethyl)trifluoroacetamide (BSTFA) / 1% trimethylchlorosilane (TMCS) at room temperature and analysed by quadrupole mass spectrometry. Ions monitored were m/z 458-465 corresponding to the m0-m8 mass isotopomers. The fractional isotopomer distribution measured was corrected for the fractional distribution due to natural abundance of ^{13}C and ^2H by multiple linear regression as described by Lee et al.³³ to obtain the excess fractional distribution of mass isotopomers resulting from isotope dilution and isotope incorporation of the administered labels. In this approach, fractional enrichment of the IV administered D7 cholesterol (M7) in the fetal tissues represents the relative transport of maternal cholesterol to the fetal tissues and was calculated by dividing the fractional enrichment of M7 within each of the respective fetal tissues by the fractional enrichment of M7 in the plasma of the mother at the time of termination (72 hours).

MIDA calculations

The MIDA (Mass Isotopomer Distribution Analysis) approach was used to estimate *de novo* cholesterol synthesis in both fetus and dam³⁴⁻³⁶. The MIDA algorithm allows us to estimate the fractional contribution of newly synthesized cholesterol from a non-accessible precursor pool. The first step of this algorithm is the calculation of the ratio of mass-isotopomer distributions of M1 and M3 due to incorporation of the precursor 1- ^{13}C -acetate. By comparing these ratios to theoretical multinomial curves, in which precursor pool enrichment is plotted against the ratios of M1 and M3, the corresponding precursor pool can be estimated. Next, from a curve in which M1 and M3 are plotted against precursor pool enrichment, mass-isotopomer distributions of the newly synthesized cholesterol can be estimated. The ratio of the measured fractional distribution of cholesterol and the estimated fractional distribution in the newly synthesized cholesterol gives the fractional contribution of newly synthesized cholesterol in the pool, and is defined as the fraction of the cholesterol pool that has been synthesized per unit of time.

RNA isolation and RT-qPCR

In total, placenta and liver from 10 *Lrp2*^{+/-} fetuses per group (that were not used for the lipid extractions), originating from five independent litters, were stored at -80 °C. RNA was extracted with TriReagent (Sigma, St. Louis, MO) to obtain total RNA fractions and quantified using a Nanodrop2000c (ThermoScientific, Pittsburgh, PA, USA). Real-time quantitative PCR were performed on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA) as previously described³⁷. The qPCR data were normalized against the geometric mean of *36b4* (*Rplp0*) and *B-actin*, (*Atcb*) performed as separate runs. Primer sequences were used as previously described³⁴⁻³⁶.

Statistics

Results of the stable isotope data and cholesterol measurements were analysed by a univariate model (Pearson), according to genotype, experimental group and litter of origin³⁸, using SPSS v20 (SPSS Inc., Chicago) or Mann–Whitney U test. Significance was assumed when $p < 0.05$ according to Tukey post hoc test or Mann–Whitney U. Significance levels are denoted as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

LRP2 does not play a quantitative role in maternal-fetal cholesterol transport during the second half of murine pregnancy

Lrp2 mice were bred heterozygously, allowing us to investigate the influence of all three possible genotypes on maternal-fetal cholesterol transport and resulting fetal cholesterol levels *in utero*. Stable isotope labeling and Mass Isotopomer Distribution Analysis (MIDA) were applied to characterize maternal-fetal cholesterol transport and determine contributions of endogenously synthesized cholesterol in the fetus. A total of 8 fetuses per genotype, originating from 5

independent litters, were analyzed within the control group. To directly compare genotypes within one litter, each litter contained at least one wild type and one knockout fetus. For the wild type and heterozygotes the average total fetal body weights were similar, while the *Lrp2*^{-/-} fetuses showed severe growth retardation ($p < 0.001$; Figure 1A). Despite the apparent difference in fetal weight, placental weights between the three genotypes were identical (Figure 1B).

Seen the previously shown expression of *Lrp2* in the maternal-fetal interfaces of the yolk sac and placenta, we analyzed whether absence of functional LRP2 protein would affect total fetal cholesterol concentrations *in utero*. Total fetal cholesterol concentrations were on average 1.7 mg/g, no differences were observed between the three genotypes. Cholesterol concentrations in the fetal brain, another known site of *Lrp2* expression, were overall higher than for other fetal tissues, averaging 2.7 mg/g, though the levels of cholesterol were not affected by genotype (Figure 1C).

Furthermore, the genotype had no influence on neither the measured fractional enrichments of D7 cholesterol (Supplementary figure 1), nor the calculated maternal contributions of cholesterol for the fetal body and brain (Figure 1D). The observed fractional synthesis rates were also unchanged in the fetal body. An exception is the fetal brain, where fractional synthesis rates within the knockouts were on average 20% increased relative to wild type and heterozygous fetuses (Figure 1E, $P = 0.02$ and $P = 0.014$, respectively).

Probucol administration during pregnancy decreases maternal plasma cholesterol and maternal-fetal cholesterol transport

Probucol was administered to half of the dams as a model for decreased cholesterol availability to the fetus during pregnancy. Parameters for the dams from the control and probucol treated group ($n = 5$ per group) are listed in Table 1, no differences in body weight, liver weight, liver cholesterol concentrations or litter size were observed. Both after one week on the probucol supplemented diet

and at E16.5 of pregnancy, total plasma cholesterol in the dams were strongly decreased relative to the control group (Figure 2A, $P < 0.001$, Mann–Whitney U). Pooled plasma samples of the dams at E16.5 were subjected to FPLC profiling, indicating that probucol treatment leads to a significant decrease of mainly HDL cholesterol, while a smaller reduction in LDL cholesterol was observed (Figure 2B).

Despite significantly decreased maternal plasma cholesterol levels, both decay curves of the D7 label after intravenous administration as well as fractional contributions of the D7 cholesterol label indicated no differences between dams from the control and probucol treated dams (Figure 3A, $p = 0.187$, repeated measurement ANOVA). The fractional contributions of newly synthesized cholesterol determined by MIDA and the estimated precursor pool enrichments were further not different between dams from the control and probucol treated group after provision of 2% $1\text{-}^{13}\text{C}$ acetate drinking water (Figure 3B+C, $p = 0.792$ and $p = 0.318$ respectively). The estimated precursor pools in the dam blood were lower in both the control and probucol supplemented group at the 8 hour time point compared to later time points, suggesting that from the 8 hour time point on steady state was achieved ($P\text{-treatment} = 0.318$).

To determine if decreased maternal plasma cholesterol levels would affect maternal-fetal cholesterol transport and fetal cholesterol levels, we measured the fractional enrichments of the D7 label for relevant fetal tissues from the control and probucol supplemented group. Fetal weights for the two groups isolated are shown in Figure 4A, no differences were observed for total fetus weights nor any of the fetal organs analyzed, i.e., brain and liver. Linear regression analysis showed that the average fractional D7 enrichments per dam were strongly correlated with maternal plasma cholesterol levels at the time of isolation (E16.5, Figure 4B, $p < 0.001$). Significant contributions of maternally derived cholesterol were evident for all fetal tissues within the control group, averaging from 50.7% in the fetal blood to 35.0% for the fetal body. Only limited contributions of maternal cholesterol were found in the fetal brain, on average 11%. Maternal contributions for fetuses from

the probucol treated group were further 50% decreased relative to control in all fetal tissues (Figure 4C, $p<0.001$). Fetal plasma cholesterol levels were determined for 5 plasma pools within the control and probucol group and were found to be on average 20% decreased in the probucol group (2.24 mmol/l and 1.80 mmol/l respectively, $p=0.032$; Figure 4E). Despite this apparent reduction in maternal and fetal plasma cholesterol concentrations upon probucol treatment, fetal tissue cholesterol levels were largely unchanged, with the exception of the fetal liver, where a slight but significant increase in cholesterol levels was observed (Figure 4D, $p=0.001$).

Decreased maternal-fetal cholesterol transport by probucol treatment is associated with increased synthesis rates in the fetal liver and blood

With maternally derived contributions of fetal cholesterol decreased and largely unchanged cholesterol levels in the fetal tissues upon probucol treatment, we turned our attention to the MIDA to determine if synthesis rates would be increased in the fetal tissues. Fractional synthesis rates as measured in the maternal blood at the time of isolation were similar between the control and probucol treated dams, averaging to 12.9%. Fractional synthesis rates as measured in the fetal tissues were much higher than in the dam, ranging from 20.9% in the fetal blood to 13.4% in the fetal brain. Synthesis rates were further slightly increased in the fetal blood (27.0%) and liver (29.0%) for fetuses from the probucol treated group relative to control, while synthesis rates were unchanged for the fetal brain (Figure 5A, $p=0.015$, $p=0.021$ and $p=0.959$ respectively). Fractional synthesis rates in the remaining fetal body were not statistically different ($p=0.130$).

Furthermore, absolute contributions of newly synthesized cholesterol in the fetal liver of both groups, calculated by multiplying tissue levels of cholesterol per gram of tissue with the FSR, showed an highly significant inverse correlation with absolute contributions of maternally derived cholesterol (Figure 5B, $r^2=0.607$, $p<0.001$). Since the assumption of a steady state condition is *per*

definitionem not valid for a growing tissue, we examined the possibility that this correlation may relate to differences in fetal liver weight, which was not the case (Figure 5B, $r^2 = 0.023$, $p = 0.578$).

Moreover, for the fetal brain, which relies mostly on endogenous synthesis during this stage of pregnancy, this correlation was absent ($r^2 = 0.091$).

Since these results could be explained both by an increase in fetal hepatic synthesis rates as well as decreased efflux, we analyzed gene expression levels of the most relevant genes involved. Overall, the transcriptional levels of the cholesterol transporters *Abca1* and *Abcg1* in the fetal liver were not different between control and the probucol treated group. Next, we measured transcription levels of several genes involved in cholesterol synthesis, none of which reached statistical significance (Figure 5C, *Hmgcr* ($p = 0.206$), *Sqle*, ($p = 0.391$) and *Mvk*, ($p = 0.268$).

Alternatively, changes in the observed FSR may relate to underlying differences in the acetyl-CoA pool, translating to changes in the observed precursor pool enrichments. However, estimated precursor pool enrichments in the fetal blood and liver were not significantly different from those in the dams' blood at the time of termination, averaging to 9.2%. Precursor pool enrichments in the fetal body and brain were lower compared to those of the fetal liver and blood (7.3% and 5.7% respectively) but no differences between the control and probucol group were observed for any of the fetal tissues (Figure 5D). Finally, theoretical distributions of maternally derived cholesterol, newly synthesized and unaccounted cholesterol were calculated for the fetal body and fetal livers from the control and probucol supplemented group. These results indicate that a larger percentage of the cholesterol pool in these tissues may be comprised of cholesterol that is not derived from the mother, or from newly synthesized (data not shown).

Decreased efflux capacity of the placenta by probucol may contribute to the observed reduced plasma cholesterol levels in the fetus

The placenta constitutes a direct interface between the maternal and fetal circulation that contributes to the transport of cholesterol to the fetus. Since probucol significantly reduced maternal-fetal cholesterol transport, we analyzed the placentas of the corresponding fetuses (n=8 per group). Placental weights are shown in Figure 6A, showing no differences between placentas of the control and probucol group (p=0.527). Cholesterol concentrations in the placenta showed a trend towards lower levels in the probucol group compared to control (Figure 6B, p=0.105). Fractional enrichments of the D7 label were similar to those observed in the maternal blood, translating to a large fractional contribution of maternally derived cholesterol, averaging to about 80% for both groups. Both fractional enrichments of the D7-cholesterol label and calculated maternal contributions were not changed for placentas of the probucol group (Figures 6C and D). Fractional synthesis rates as determined by MIDA were similar to those observed in the maternal blood at E16.5, averaging to about 11%, with no apparent differences between the control and probucol group.

Since only a trend towards reduced cholesterol levels was observed in the probucol supplemented group and contributions of maternally derived cholesterol were found to be similar between the two groups, we analyzed transcript levels of several key lipoprotein receptors mediating cholesterol uptake in the placental trophoblast. Expression of the low density lipoprotein receptor (*Ldlr*) was significantly increased for placentas from the probucol treated group, while transcript levels of scavenger receptor class B type I (*Srb1*) were unchanged (Figure 6F, p=0.019 and p=0.743 respectively).

Additionally, we analyzed transcript levels for several transporters from the ATP-binding cassette family and phospholipid transfer protein (*Pltp*), all of which have been implemented in mediating efflux of cholesterol to the fetal circulation. Although gene expression levels for *Abca1* were slightly

decreased in the probucol supplemented group and unchanged for *Abcg1*, transcript levels for *Pltp* were significantly decreased upon probucol treatment ($p=0.005$).

Heart anomalies associated with LRP2 knockout do not relate to local cholesterol uptake or maternal cholesterol levels

Lrp2 is expressed in several tissues contributing to the development of the fetal heart. Consequently, *Lrp2*^{-/-} fetuses show a range of severe cardiac abnormalities that may relate to cholesterol availability *in utero*, especially common arterial trunk and ventricular myocardial thinning²⁴. Local uptake of cholesterol, presented as the fractional enrichments of the IV administered D7 label, is shown in Figure 7A. Even when standardized to enrichments in the wild type fetal hearts within the same litter, fractional enrichments showed no differences between the three genotypes (ANOVA, $p=0.642$).

Finally, we tested the possibility that strongly reduced maternal plasma cholesterol levels during pregnancy may induce heart defects in the otherwise normally developing *Lrp2*^{+/-} fetuses. Histology of the fetal heart for both the control and probucol supplemented groups revealed no indication for disturbed cardiac development, showing normal septation of the outflow tract and development of the ventricular septa (Figure 7B). Furthermore, no differences were observed in the thickness of the ventricular myocardium for the probucol treated fetuses relative to the control group ($p=0.418$, Figure 7C).

Discussion

Low density lipoprotein-related receptors (LRPs) comprise a group of multifunctional endocytic cell surface receptors, likely to have acquired complex evolutionary intertwined functions between lipid metabolism and developmentally regulated signaling pathways³⁹.

LRP2 is the largest receptor of this family. It is implemented in the internalization of a large variety of ligands of potential importance during pregnancy, including cholesterol containing lipoprotein particles^{20,40}. Although *Lrp2* is expressed on maternal-fetal interfaces of both the yolk sac and the placenta during human and murine pregnancy, a quantitative role in maternal-fetal cholesterol transport has not been investigated⁴¹. Using a heterozygous breeding approach in combination with stable isotope labeling, we aimed to quantify the effect of absence of LRP2 during pregnancy on maternal-fetal cholesterol transport and the resulting cholesterol levels in the mouse fetus.

Overall, no differences were found in the relative abundance of labeled cholesterol or calculated maternal contributions between *Lrp2* genotypes in our mouse model. Furthermore, despite a significant effect of LRP2 knockout on fetal body weight, genotype had no discernible influence on fetal cholesterol levels or calculated synthesis rates *in utero*. An exception was the fetal *Lrp2*^{-/-} brain, which showed higher fractional synthesis rates compared to those of wild type and heterozygous.

The low fractional D7 enrichments found in the fetal brain concur with a strong dependence on endogenous cholesterol synthesis for the fetal brain at this stage and were further not affected by genotype. This suggests that the contributing effect of maternal-fetal transport is likely negligible for brain cholesterol levels and that loss of *Lrp2* expression in the developing nervous system itself gives rise to the observed neural tube defects, severely affecting further brain development^{22,42,43}.

This suggests that the observed increase in cholesterol synthesis rates are likely the result of structural differences in the *Lrp2*^{-/-} brain that may relate to underlying differences in cell populations, having different synthesis and/or turnover rates, or (dys)functioning of the blood-brain-barrier itself. Together, these results imply that the mass transport of maternal cholesterol to the mouse fetus

does not critically depend on the presence of functional LRP2 during this time of pregnancy. This suggests that either transport of alternate ligands or defects in local signaling pathways during development are a more likely cause of the observed heart and brain defects^{22,40,42}.

In addition to studying the effect of genotype on maternal-fetal cholesterol transport, we used probucol as a model for decreased cholesterol availability to the fetus. Analysis of the maternal blood confirmed that probucol treatment was effective in reducing plasma cholesterol levels in the dam with more than 60% during pregnancy, mainly by reduction of HDL cholesterol. Since a significant part of the fetal cholesterol is derived from the mother during pregnancy, we anticipated a reduction in maternal-fetal cholesterol transport as well as a potential interaction with the *Lrp2* genotype.

In line with previous research in mice, significant fractional contributions of labeled cholesterol were found in fetal tissues, indicating that a large part of the fetal cholesterol pool is indeed maternally derived⁴. Maternal contributions were further significantly lowered for the probucol treated group, suggesting that probucol was successful in reducing maternal-fetal cholesterol transport. Since the fetal mouse brain is considered to rely mostly on endogenous cholesterol synthesis at this stage of pregnancy, only limited maternal cholesterol contributions were found, corresponding to values found previously⁴.

Interestingly, despite the observed reduction of maternal-fetal cholesterol transport for the probucol treated fetuses, overall cholesterol levels and synthesis rates as measured in the placenta were not significantly changed compared to placentas of the control group. In the current view of placental cholesterol metabolism, the placenta largely relies on the uptake of maternally derived cholesterol. Although we have not explicitly addressed endogenous placental cholesterol synthesis, our data do not give indications that it plays a significant role in our murine model system. This view is supported by the multitude of different lipoprotein receptors found to be expressed on the apical side of the placental syncytiotrophoblast and the high maternal contributions found in our model⁴⁴.

Although it is uncertain to what extent esterified cholesterol from the placental pool exchanges with maternally derived cholesterol, one interpretation of these results would be that the placenta, at the administered dose of probucol, can still compensate for the decrease in maternal cholesterol availability by increasing transport into the trophoblast through upregulation of lipoprotein receptors, in order to maintain cholesterol homeostasis. This view is supported by the association with the increased expression of the placental Low Density Lipoprotein receptor (LDLR) in our model, which is considered, together with SR-BI, as a key lipoprotein receptor within the placental trophoblast⁴⁵⁻⁴⁸. This may suggest that in mice extra-embryonic tissues may take up more LDL cholesterol from the maternal blood when maternal HDL concentrations are low.

Efflux of placental cholesterol to the fetal circulation is thought to be mediated by endothelial cells on the fetal side of the placenta, potentially mediated by the ABC transporters, ABCG1 and ABCA1²⁹.

Probucol supposedly acts by selective inhibition and inactivation of ABCA1 in the plasma membrane but has limited effects on ABCA1 transcript levels itself^{49,50}. Although the exact absolute contributions of the yolk sac and placenta in cholesterol transport to the fetus remain to be accurately determined throughout murine pregnancy, probucol mediated inhibition of efflux to fetal HDL through ABCA1 is likely contributing to the observed reduction in maternal-fetal cholesterol transport and lowered fetal plasma cholesterol levels⁴⁸.

In addition to a role for ABC transporters, endothelial cells facing the fetal circulation are a major site of phospholipid transfer protein (PLTP) expression, likely to secrete active PLTP to promote cholesterol efflux from cells to HDL-apolipoproteins and HDL acceptors^{51,52}. The reduced expression levels of placental *Pltp* found might further add to the idea that probucol, either directly or indirectly, interferes with the efflux capacity of the placenta, corresponding to previously performed *in vitro* studies²⁹.

Interestingly, despite a strong decrease in the contributions of maternal cholesterol, probucol, treatment lead to a significant increase in hepatic cholesterol levels in the mouse fetus, whereas cholesterol levels in the remaining fetal body and brain were unchanged. Furthermore, endogenous cholesterol synthesis, measured using MIDA, indicated that contributions of newly synthesized cholesterol in the fetal liver and fetal blood were modestly increased upon treatment with probucol. Since it is currently unknown whether probucol can cross the placenta and its presence cannot be deduced by measuring transcript levels of *Abca1* itself, we speculate that either inhibition of ABCA1 in the fetal liver and/or placenta may explain the observed reduction of maternally derived cholesterol and increased contributions of newly synthesized cholesterol in our model.

High fetal growth rates during the end of gestation likely induce a higher demand for fetal cholesterol, where endogenous synthesis provides a large part of the fetal cholesterol, as is reflected by high expression and activity of genes involved in cholesterol synthesis^{53,54}. At the same time, this represents one of the caveats of the MIDA approach, since this method assumes that cholesterol exchange is in a steady state situation, which can never be the case in a rapidly growing fetus. Despite these limitations, absolute contributions of newly synthesized cholesterol in the fetal liver showed a highly significant, inverse correlation with the contribution of maternally derived cholesterol. This association could not be explained by differences in individual liver weights, representing the total cholesterol pool, nor could be related to underlying differences in observed precursor pool enrichments. Together these results suggest that the mouse fetus can most likely counteract for a decrease in maternally derived cholesterol by a compensatory increase in cholesterol synthesis rates in fetal tissues, especially the fetal liver, as has been suggested before^{55,56}.

Strikingly, these results are reminiscent of previous studies using LXR agonists during murine pregnancy, that lead to increased maternal-fetal cholesterol transport. Furthermore, these studies showed increased fetal plasma cholesterol levels and lowered hepatic cholesterol levels that were

most likely caused by upregulation of *Abca1* within the fetal-placental axis ^{37,48}. Our data and previous studies provide reasonable indications that excretory pathways to fetal HDL are already functional in the fetal mouse liver and would implement an important role for placental and fetal ABCA1 in this process ^{37,57}. Although interpretation of our data is hampered by a non-steady state condition and missing information about absolute fetal growth rates during the time of measurement, a possibility is that the reduced maternal contributions upon probucol treatment reflect a decrease in maternal-fetal exchange of cholesterol instead of reflecting net import, as this could possibly account for the missing cholesterol in our model. Considering that ABCA1 is expressed in multiple compartments of the murine placenta, this is an interesting possibility for further studies.

Finally, in addition to a role for LRP2 in mediating maternal-fetal cholesterol transport, results from previous studies have shown that *Lrp2* is expressed in several tissues contributing to the development of the fetal heart ^{21,58}. We have recently extended these observations by demonstrating that *Lrp2* is expressed in all cell populations involved in the formation and septation of the outflow tract and compaction of the ventricular myocardium ²⁴. Consequently, *Lrp2*^{-/-} fetuses show a near complete penetrance of severe cardiac defects, that we hypothesized to originate from a reduced ability for cholesterol uptake within any of the cell populations involved ²⁴. However, since fractional enrichments of the labeled cholesterol in the fetal heart were shown to be independent of the *Lrp2* genotype, our data cannot exclude the possibility that ligands other than cholesterol are related to the observed cardiac anomalies.

Additionally, we examined the possibility that heterozygosity for *Lrp2*, in combination with strongly reduced maternal cholesterol levels by probucol treatment, would interact to induce heart defects in the heterozygous offspring. Despite a significant reduction of maternally derived cholesterol by probucol in fetal tissues, we observed none of the cardiac anomalies present in the *Lrp2*^{-/-} offspring. These results, in our view, support the idea that the cardiac anomalies, observed in the *Lrp2*^{-/-}

fetuses, are not related to the concentration of cholesterol but more likely the result of disturbed signaling or uptake of other potentially important ligands, which has been suggested previously^{22,42}

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Conflict of interest

-none-

Abbreviations:

LRP2 – Low Density Lipoprotein Related Receptor 2
SHH – Sonic HedgeHog
Probucol - 4,4'-[propane-2,2-diylbis(thio)]bis(2,6-di-tert-butylphenol)
HDL – High Density Lipoprotein
LDL – Low Density Lipoprotein
IDL – Intermediate Density Lipoprotein
VLDL – Very Low Density Lipoprotein
ABCA1 – ATP Binding Cassette type A1
ABCG1 – ATP Binding Cassette type G1
LDLR – Low Density Lipoprotein Receptor
HMGCR - 3-hydroxy-3-methylglutaryl-CoA reductase
SQLE - Squalene Epoxidase
MVK - Mevalonate Kinase
PLTP - Phospholipid Transfer Protein
SR-BI - Scavenger Receptor Class B member 1
LXR - Liver X Receptor
E13.5 – Embryonic Day 13.5
EDTA - Ethylenediaminetetraacetic acid
PBS - Phosphate Buffered Saline
FPLC - Fast Protein Liquid Chromatography
RT-qPCR – Reverse Transcription Quantitative Polymerase Chain Reaction
D7 cholesterol - Cholesterol-25,26,26,26,27,27,27-d
1-13C acetate - Sodium acetate-1-13C
MIDA – Mass Isotopomer Distribution Analysis
GC/MS - Gas Chromatography – Mass Spectrometry

BSTFA - N,O-bis-(trimethyl)trifluoroacetamide
 TMCS – Trimethylchlorosilane
 FSR – Fractional Synthesis Rate
 SD – Standard Deviation

Table 1. Dam parameters at hysterectomy

	Control (n=5)	Probucol (n=5)
Body weights (g)	31.7 +/- 2.6	35.3 +/- 3.9
Liver weight (g)	1.53 +/- 0.24	1.64 +/- 0.24
Liver weight/BW (%)	4.84 +/- 0.69	4.64 +/- 0.47
Cholesterol liver (mg/g)	2.67 +/- 0.13	2.58 +/- 0.18
Litter size (n)	7.8 +/- 3.3	8.6 +/- 1.3

Table 1. Parameters for the pregnant dams from the control and probucol treated group at embryonic day 16 (E16.5), used for the stable isotope experiments. None of the parameters were statistically significant between the control and probucol treated dams. Data represent mean +/- SD for 5 dams per group.

References

1. Napoli C, D'armiento F, Mancini F, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest.* 1997;100(11):2680.
2. Amaral C, Gallardo E, Rodrigues R, et al. Quantitative analysis of five sterols in amniotic fluid by GC–MS: Application to the diagnosis of cholesterol biosynthesis defects. *Journal of Chromatography B.* 2010;878(23):2130-2136.
3. Baardman ME, Erwich JJH, Berger RM, et al. The origin of fetal sterols in second-trimester amniotic fluid: Endogenous synthesis or maternal-fetal transport? *Obstet Gynecol.* 2012;207(3):202. e19-202. e25.
4. Tint G, Yu H, Shang Q, Xu G, Patel SB. The use of the Dhcr7 knockout mouse to accurately determine the origin of fetal sterols. *J Lipid Res.* 2006;47(7):1535-1541.
5. Tint GS, Irons M, Elias ER, et al. Defective cholesterol biosynthesis associated with the smith-lemli-opitz syndrome. *N Engl J Med.* 1994;330(2):107-113.

6. Kelley RI, Roessler E, Hennekam R, et al. Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: Does abnormal cholesterol metabolism affect the function of sonic hedgehog? *Am J Med Genet.* 1996;66(4):478-484.
7. Schell-Apacik C, Rivero M, Knepper JL, Roessler E, Muenke M, Ming JE. SONIC HEDGEHOG mutations causing human holoprosencephaly impair neural patterning activity. *Hum Genet.* 2003;113(2):170-177.
8. Dyer LA, Kirby ML. Sonic hedgehog maintains proliferation in secondary heart field progenitors and is required for normal arterial pole formation. *Dev Biol.* 2009;330(2):305-317.
9. Edison RJ, Berg K, Remaley A, et al. Adverse birth outcome among mothers with low serum cholesterol. *Pediatrics.* 2007;120(4):723-733.
10. Sattar N, Greer IA, Galloway PJ, et al. Lipid and lipoprotein concentrations in pregnancies complicated by intrauterine growth restriction. *Journal of Clinical Endocrinology & Metabolism.* 1999;84(1):128-130.
11. Pecks U, Caspers R, Schiessl B, et al. The evaluation of the oxidative state of low-density lipoproteins in intrauterine growth restriction and preeclampsia. *Hypertension in Pregnancy.* 2012;31(1):156-165.
12. Smedts H, van Uitert E, Valkenburg O, et al. A derangement of the maternal lipid profile is associated with an elevated risk of congenital heart disease in the offspring. *Nutrition, Metabolism and Cardiovascular Diseases.* 2012;22(6):477-485.
13. Toescu V, Nuttall S, Martin U, et al. Changes in plasma lipids and markers of oxidative stress in normal pregnancy and pregnancies complicated by diabetes. *Clin Sci.* 2004;106(1):93-98.
14. Woollett L. Review: Transport of maternal cholesterol to the fetal circulation. *Placenta.* 2011;32:S218-S221.
15. Burke KT, Colvin PL, Myatt L, Graf GA, Schroeder F, Woollett LA. Transport of maternal cholesterol to the fetus is affected by maternal plasma cholesterol concentrations in the golden syrian hamster. *J Lipid Res.* 2009;50(6):1146-1155.
16. Santander NG, Contreras-Duarte S, Awad MF, et al. Developmental abnormalities in mouse embryos lacking the HDL receptor SR-BI. *Hum Mol Genet.* 2013;22(6):1086-1096.
17. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest.* 1993;92(2):883.
18. Kantarci S, Al-Gazali L, Hill RS, et al. Mutations in LRP2, which encodes the multiligand receptor megalin, cause donnai-barrow and facio-oculo-acoustico-renal syndromes. *Nat Genet.* 2007;39(8):957-959.
19. Marzolo M, Farfán P. New insights into the roles of megalin/LRP2 and the regulation of its functional expression. *Biol Res.* 2011;44(1):89-105.
20. Assémat E, Châtelet F, Chandellier J, et al. Overlapping expression patterns of the multiligand endocytic receptors cubilin and megalin in the CNS, sensory organs and developing epithelia of the rodent embryo. *Gene expression patterns.* 2005;6(1):69-78.
21. Willnow TE, Hilpert J, Armstrong SA, et al. Defective forebrain development in mice lacking gp330/megalyn. *Proceedings of the National Academy of Sciences.* 1996;93(16):8460-8464.
22. Spoelgen R, Hammes A, Anzenberger U, et al. LRP2/megalyn is required for patterning of the ventral telencephalon. *Development.* 2005;132(2):405-414.

23. Hammes A, Andreassen TK, Spoelgen R, et al. Role of endocytosis in cellular uptake of sex steroids. *Cell*. 2005;122(5):751-762.
24. Baardman ME, Zwier MV, Wisse LJ, et al. Common arterial trunk and in Lrp2 knock out mice indicate a crucial role of LRP2 in cardiac development. *Disease Models and Mechanisms*. 2016;dmm. 022053.
25. Yoshikawa T, Shimano H, Chen Z, Ishibashi S, Yamada N. Effects of probucol on atherosclerosis of apoE-deficient or LDL receptor-deficient mice. *Hormone and Metabolic Research*. 2001;33(08):472-479.
26. Timmins JM, Lee J, Boudyguina E, et al. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest*. 2005;115(5):1333-1342.
27. Isomaa B, Almgren P, Tuomi T, et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care*. 2001;24(4):683-689.
28. Baumann M, Körner M, Huang X, Wenger F, Surbek D, Albrecht C. Placental ABCA1 and ABCG1 expression in gestational disease: Pre-eclampsia affects ABCA1 levels in syncytiotrophoblasts. *Placenta*. 2013;34(11):1079-1086.
29. Stefulj J, Panzenboeck U, Becker T, et al. Human endothelial cells of the placental barrier efficiently deliver cholesterol to the fetal circulation via ABCA1 and ABCG1. *Circ Res*. 2009;104(5):600-608.
30. Gundersen H, Bendtsen T, KORBO L, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS*. 1988;96(1-6):379-394.
31. Neese R, Faix D, Kletke C, et al. Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA. *American Journal of Physiology-Endocrinology And Metabolism*. 1993;264(1):E136-E147.
32. Bligh E, Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*. 1959;37(8):911-917.
33. Lee WP, Byerley LO, Bergner EA, Edmond J. Mass isotopomer analysis: Theoretical and practical considerations. *Biol Mass Spectrom*. 1991;20(8):451-458.
34. Hellerstein M, Neese R. Mass isotopomer distribution analysis: A technique for measuring biosynthesis and turnover of polymers. *American Journal of Physiology-Endocrinology And Metabolism*. 1992;263(5):E988-1001.
35. Oosterveer MH, van Dijk TH, Tietge UJ, et al. High fat feeding induces hepatic fatty acid elongation in mice. *PLoS One*. 2009;4(6):e6066.
36. van Meer H, van Straten EM, Baller JF, et al. The effects of intrauterine malnutrition on maternal-fetal cholesterol transport and fetal lipid synthesis in mice. *Pediatr Res*. 2010;68(1):10-15.
37. van Straten EM, Huijkman NC, Baller JF, Kuipers F, Plösch T. Pharmacological activation of LXR in utero directly influences ABC transporter expression and function in mice but does not affect adult cholesterol metabolism. *American Journal of Physiology-Endocrinology And Metabolism*. 2008;295(6):E1341-E1348.
38. Festing MF. Design and statistical methods in studies using animal models of development. *ILAR Journal*. 2006;47(1):5-14.
39. Bazan JF, Janda CY, Garcia KC. Structural architecture and functional evolution of wnts. *Developmental cell*. 2012;23(2):227-232.

40. Marzolo M, Farfán P. New insights into the roles of megalin/LRP2 and the regulation of its functional expression. *Biol Res.* 2011;44(1):89-105.
41. Fisher CE, Howie SE. The role of megalin (LRP-2/Gp330) during development. *Dev Biol.* 2006;296(2):279-297.
42. Kur E, Mecklenburg N, Cabrera RM, Willnow TE, Hammes A. LRP2 mediates folate uptake in the developing neural tube. *J Cell Sci.* 2014;127(Pt 10):2261-2268.
43. Gajera CR, Emich H, Lioubinski O, et al. LRP2 in ependymal cells regulates BMP signaling in the adult neurogenic niche. *J Cell Sci.* 2010;123(11):1922-1930.
44. Woollett LA. Maternal cholesterol in fetal development: Transport of cholesterol from the maternal to the fetal circulation. *Am J Clin Nutr.* 2005;82(6):1155-1161.
45. Wadsack C, Hammer A, Levak-Frank S, et al. Selective cholesteryl ester uptake from high density lipoprotein by human first trimester and term villous trophoblast cells. *Placenta.* 2003;24(2):131-143.
46. Grimes RW, Pepe GJ, Albrecht ED. Regulation of human placental trophoblast low-density lipoprotein uptake in vitro by estrogen. *J Clin Endocrinol Metab.* 1996;81(7):2675-2679.
47. Hatzopoulos AK, Rigotti A, Rosenberg RD, Krieger M. Temporal and spatial pattern of expression of the HDL receptor SR-BI during murine embryogenesis. *J Lipid Res.* 1998;39(3):495-508.
48. Lindegaard ML, Wassif CA, Vaisman B, et al. Characterization of placental cholesterol transport: ABCA1 is a potential target for in utero therapy of Smith–Lemli–Opitz syndrome. *Hum Mol Genet.* 2008;17(23):3806-3813.
49. Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probucol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler Thromb Vasc Biol.* 2004;24(12):2345-2350.
50. Wu CA, Tsujita M, Hayashi M, Yokoyama S. Probucol inactivates ABCA1 in the plasma membrane with respect to its mediation of apolipoprotein binding and high density lipoprotein assembly and to its proteolytic degradation. *J Biol Chem.* 2004;279(29):30168-30174.
51. Oram JF, Wolfbauer G, Vaughan AM, Tang C, Albers JJ. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. *J Biol Chem.* 2003;278(52):52379-52385.
52. Scholler M, Wadsack C, Metso J, et al. Phospholipid transfer protein is differentially expressed in human arterial and venous placental endothelial cells and enhances cholesterol efflux to fetal HDL. *The Journal of Clinical Endocrinology & Metabolism.* 2012;97(7):2466-2474.
53. Carr BR, Simpson ER. Cholesterol synthesis in human fetal tissues*. *The Journal of Clinical Endocrinology & Metabolism.* 1982;55(3):447-452.
54. Belknap WM, Dietschy JM. Sterol synthesis and low density lipoprotein clearance in vivo in the pregnant rat, placenta, and fetus. sources for tissue cholesterol during fetal development. *J Clin Invest.* 1988;82(6):2077-2085.
55. Jurevics HA, Kidwai FZ, Morell P. Sources of cholesterol during development of the rat fetus and fetal organs. *J Lipid Res.* 1997;38(4):723-733.

56. Woollett LA. Origin of cholesterol in the fetal golden syrian hamster: Contribution of de novo sterol synthesis and maternal-derived lipoprotein cholesterol. *J Lipid Res.* 1996;37(6):1246-1257.
57. Aye I, Waddell B, Mark P, Keelan J. Oxysterols inhibit differentiation and fusion of term primary trophoblasts by activating liver X receptors. *Placenta.* 2011;32(2):183-191.
58. Sahali D, Mulliez N, Chatelet F, et al. Comparative immunochemistry and ontogeny of two closely related coated pit proteins. the 280-kd target of teratogenic antibodies and the 330-kd target of nephritogenic antibodies. *Am J Pathol.* 1993;142(5):1654-1667.

Figure legends:

Figure 1. Overview of the stable isotope labeling results to characterize maternal fetal cholesterol transport and fetal cholesterol levels depending on *Lrp2* genotype. Data for fetuses from the control group is shown, according to genotype. The influence of genotype on the resulting fetal (A) and placental weights (B) are shown. (C) Cholesterol concentrations as measured in the fetal tissues, white bars represent the results for the *Lrp2*^{+/+} fetuses, grey bars *Lrp2*^{+/-} and black bars *Lrp2*^{-/-}. (D) Contributions of maternally derived cholesterol as calculated from fractional D7 enrichments in the dam blood and fetal tissues. (E) Fractional contributions of newly synthesized cholesterol as determined by Mass Isotopomer Distribution Analysis (MIDA). In total, 24 fetuses were analyzed according to genotype (n=8 each). Each litter contained at least one wild type and one knockout fetus. Data represent mean +/- SD.

Figure 2. (A) Total plasma cholesterol levels for the dams after 1 week on the control and probucol supplemented diet (prior to mating) and during pregnancy, white bars represent dams from the control group, black bars the probucol supplemented group. (B) Fast Protein Liquid Chromatography (FPLC) profiles of pooled plasma samples of the pregnant dams from the control (solid line) and probucol supplemented (dotted line) group at day 16 of pregnancy. Data represent mean +/- SD.

Figure 3. (A) Fractional enrichment of D7-labeled cholesterol in the maternal blood over 72 hours after intravenous administration. Data points represent actual measurements +/- SD, lines represent the averages of individual dam curve fittings in SAAMII, of dams from the control (continuous line) and probucol treated groups (dotted line) (B). Fractional contributions of newly synthesized cholesterol in the dam blood after provision of 2% 1-¹³C acetate drinking water. (C) Estimated fractional precursor pool enrichments for dams from the control and probucol supplemented diet, measured at six different time points after provision of 2% 1-¹³C acetate in the drinking water. At the 8 hour time point, fractional precursor enrichments were significantly lower compared to later time points for both groups, (2-way repeated measurement ANOVA) while overall no differences were observed between the two groups (p= 0.318), suggesting a steady state condition over the time measured. Data represent mean +/- SD, n=5 dams per group.

Figure 4. Characterization of maternal-fetal cholesterol transport using stable isotope labeling and cholesterol levels for fetuses within the control and probucol supplemented group. (A) Weights for the analysed fetal organs isolated at E16.5, error bars represent the SD of the individual total fetus weights. (B) Regression plot showing the average fractional D7 enrichments for the fetuses plotted against the plasma cholesterol concentration in the dams of the control and probucol supplemented group (n=10). (C) Calculated fractional contributions of maternal cholesterol in the fetal blood and organs. (D) Cholesterol levels in the fetal organs,

white bars represent fetuses from the control group, black bars fetuses from the probucol group. (E) Plasma cholesterol levels for fetuses from the control (open bar) and probucol treated (filled bar) group (n=5 pools per group). All data represent mean +/- SD

Figure 5. Contributions of newly synthesized cholesterol within the fetus shown for both the control and probucol treated groups, calculated by Mass Isotopomer Distribution Analysis (MIDA). (A) Fractional synthesis rates as calculated for the dam blood at E16.5 and fetal organs, defined as the fraction of the cholesterol pool synthesized per unit of time. White bars represent fetuses from the control group, black bars the probucol supplemented group. (B) Scatter plot and linear regression of the absolute contributions of newly synthesized cholesterol against the absolute contributions of maternally derived cholesterol for the individual fetal livers from the control and probucol treated groups (Calculated by multiplying the fractional contributions of newly synthesized and maternally derived cholesterol with the cholesterol concentrations in the individual fetal liver samples, n=16). (C) Relative gene expression levels for the fetal liver. (D) Estimated precursor pool enrichments as observed in the dam blood at E16.5 and fetal organs. All data are shown as mean +/- SD.

Figure 6. Analysis results for the placentas of the fetuses from the control (white bars) and probucol (black bars) treated group at E16.5 (*Lrp2*^{+/-}, n=8 per group). (A) Average placental weight, (B) cholesterol levels, (C) fractional D7 enrichments, (D) calculated fractional contributions of maternally derived cholesterol and (E) fractional contributions of newly synthesized cholesterol. (F) Gene expressions for *Lrp2*^{+/-} placentas from the control and probucol treated group (n=10 per group).

Figure 7. Cholesterol uptake within the fetal heart per genotype and cardiac phenotype of the *Lrp2*^{+/-} mice with and without probucol treatment. (A) Fractional enrichments of the D7 administered label, standardized to fractional enrichments for the hearts of wild type fetuses within the same litter. (B) Myocardial Light Chain-2a (Mlc2a) staining as myocardial marker of global cardiac development. No differences were observed in the outflow tract region (OFT), there is normal septation of the aorta (Ao) and pulmonary trunk (Pt). Both groups display an intact ventricular septum around the mitral valve (MV) and tricuspid valve (TV), without anomalies of the atrioventricular valves. (C) Ratios of compact myocardium versus total myocardium for fetuses from the control and probucol treated group. All data represent mean +/- SD.







